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Properties of Inducible Kynureninase of *Neurospora crassa**

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The inducible kynureniase, which was purified to homogeneity and crystallized from *Neurospora crassa*, has a molecular weight of about 105,000 and consists of two subnits identical in molecular weight (50,000) and in amino terminal amino acid (1.8 moles of valine/mole of enzyme). The enzyme exhibits absorption mzxima at 280 and 430 nm, and emits fluorescent light with the maxima at 338 and 495 nm, respectively, when excited at the wavelength of the absorption maxima. One mole of pyridoxal 5′-phosphate is bound per mole of enzyme. The holoenzyme is resolved to the apoenzyme by incubation with hydroxylamine followed by dialysis, and reconstituted by addition of pyridoxal 5′-phosphate.

KEY WORDS: Inducible kynureninase/ *Neurospora crassa*/ Pyridoxal 5′-phosphate/ L-Kynurenine/

INTRODUCTION

Kynureninase (L-kynurenine hydrolase, EC 3.7.1.3) is a pyridoxal 5′-phosphate (pyridoxal-P) enzyme that catalyzes the hydrolysis of kynurenine to alanine and anthranilate. Since its discovery by Kotake and Nakayama in a mammalian liver,[1] the enzyme has been widely demonstrated in bacteria,[2~4] fungi,[5~8] and higher animals,[9~14] and is regarded as a key-enzyme of the aromatic and NAD pathways in tryptophan metabolism. Bacterial kynureninase, which is formed inducibly, has been purified to homogeneity and crystallized from *Pseudomonas fluorescens* in this laboratory.[15,16] The enzyme activity is controlled by transamination of the coenzyme moiety catalyzed by the enzyme itself.[17,18] Gaertner *et al.*[19] showed the occurrence of two types of kynureninase, *i.e.* inducible kynureninase and constitutive one (or hydroxykynureninase) in *Neurospora crassa*, which were separated by DEAE-cellulose chromatography. The enzymes differ kinetically in their responses to L-kynurenine and L-3-hydroxykynurenine. In an attempt to make a comparative study of both types of kynureninase, we have obtained a homogeneous and crystalline preparation of the inducible enzyme of *Neurospora crassa*.[20] We here report more detailed studies on enzymological and physicochemical characteristics of the inducible kynureninase of *Neurospora crassa*.

EXPERIMENTAL PROCEDURES

Materials. L-Kynurenine and D-kynurenine were synthesized from L- and D-

* This paper was accepted on the occasion of the retirement of Prof. emeritus T. Yamamoto, and was dedicated to him.

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tryptophan, respectively, by the method of Warnell and Berg.[21] *N'*-Formyl-L-kynurenine was prepared from L-kynurenine with formic-acetic anhydride according to the method of Dalgliesh.[22] L-3-Hydroxykynurenine was obtained from Calbiochem, San Diego, Calif., U.S.A.; pyridoxal-P from Kyowa Hakko Kogyo, Tokyo; L-amino acids from Ajinomoto, Tokyo; and 3-methyl-2-benzothiazolone hydrazone HCl (MBTH) from Aldrich Chemical Co., Wis., U.S.A. Dansyl chloride and dansyl amino acids were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.; and 2, 4-dinitrofluorobenzene, dinitrophenyl amino acids, specially prepared sodium dodecyl sulfate (SDS) and HCl for protein research from Wako Chemicals, Osaka. The other chemicals were analytical grade reagents.

Enzyme Preparation and Assay. The enzyme was purified to homogeneity and crystallized from a cell-free extract of *Neurospora crassa* (IFO 6068) as described previously.[20] Kynureninase was assayed by measuring the rate of decrease in the absorbance at 360 nm due to hydrolysis of kynurenine.[20] The hydrolyses of L-3-hydroxykynurenine and *N'*-formyl-L-kynurenine were determined by measuring decrease in the absorbance at 375 (ε=3,180, pH 8.5) and 321 nm (ε=3,750, pH 8.5), respectively. One unit of the enzyme was defined as the amount of enzyme required to hydrolyze 1 μmol of kynurenine per min. Specific activity was expressed as units per mg of protein.

Protein Determination. Protein was determined by measuring the absorbance at 280 nm. The absorbance coefficient of the enzyme ($A_{1\,cm}^{1\%}$=6.94) was used throughout, obtained by absorbance and dry weight determination.

NH_2-Terminal Analysis. The dialyzed enzyme (1.3 mg) was dansylated and hydrolyzed in 6 N HCL according to the procedure of Gray.[23] Dansyl amino acids in the hydrolysates were identified by two-dimensional chromatography[24] on polyamide thin layer sheets. The NH_2-terminal amino acids were also determined by the dinitrophenyl (DNP) method.[25] DNP-protein was prepared by incubation of 4.6 mg of enzyme with an equal amount of sodium bicarbonate and excess 5% 2, 4-dinitrofluorobenzene (in 99.5% ethanol) in a final volume of 1 ml at 37°C for 16 h with occasional shaking. The resultant DNP protein was hydrolyzed in 6 N HCl at 105°C for 12 h in an evacuated and sealed tube. DNP-amino acids were separated by two-demensinal paper chromatography[26] and eluted with 1% sodium bicarbonate. The absorbance was measured at 360 nm. The recoveries of DNP-amino acids were calculated according to the method of Levy[27] with internal standards.

Spectrophotometry. Spectrophotometric measurements were made with a Shimadzu MPS–50L recording spectrophotometer with a 1.0-cm light path. Fluorescence measurements were performed with a Hitachi spectrofluorophotometer type MPF–4 with a 1.0-cm light path.

RESULTS

1. Molecular Weight

In the sedimentation velocity experiments, the sedimentation constant ($S_{20,w}$) was determined to be 6.48, 6.40, and 6.32 S at the enzyme concentrations of 0.114, 0.248 and 0.339%, respectively. By extrapolation to zero protein concentration the sedimentation coefficient ($S^0_{20,w}$) was calcuated to be 6.55 S as described previously.[20] The molecular weight of the enzyme was determined to be about 105,000 by the gel filtration method.[20] The molecular weight was also estimated by electrophoresis in polyacrylamide gels of graded porosity (4–30%) according to the method of Andersson *et al.*,[28] with catalase (Mr, 244,000), yeast alcohol dehydrogenase (126,000), bacterial kynureninase (91,000[16]), bovine serum albumin (67,000), and egg albumin (45,000) as standard proteins. A molecular weight of approximately 103,000 was obtained.

2. Structure of Subunit

The enzyme was found to be completely inhibited by 1% SDS in 10 mM sodium phosphate buffer (pH 7.2) when incubated at 37°C for 1 h. No activity was recovered by dialysis against two changes of 1000 volumes of 10 mM potassium phosphate buffer

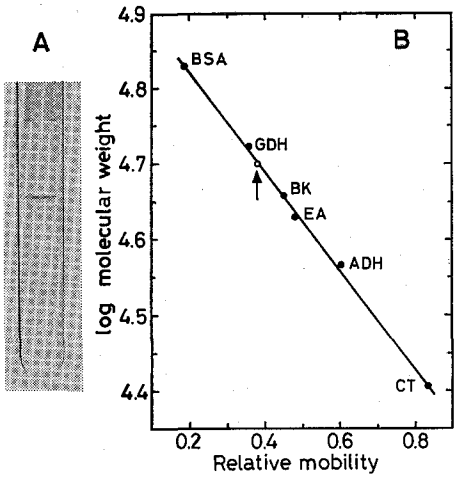

Fig. 1. A, SDS-polyacrylamide disc gel electrophoresis of fungal kynureninase. A 7-cm gel was loaded with 8 μg of the SDS-treated enzyme and electrophoresis was carried out by the method of Weber and Osborn,[29] except that a 5-mm spacer gel (2.5% acrylamide-0.6% methylenebisacrylamide) was placed on the running gel. The gel was stained with Coomassie brilliant blue. B, Determination of the subunit molecular weight. Protein standards represented by closed circles were; BSA, bovine serum albumin; GDH, glutamate dehydrogenase; BK, bacterial kynureninase; EA, egg albumin; ADH, yeast alcohol dehydrogenase; CT, chymotrypsinogen. The relative mobility of the *N. crassa* kynureninase is shown by the open circle.

(pH 7.2) at 4°C. These suggest that the denaturant causes irreversible conformational changes in the enzyme, probably with dissociation into subnits.

The subnit structure of the enzyme was examined by disc gel electrophoresis. The enzyme was incubated with 1.0% SDS in 50 mM sodium phosphate buffer (pH 7.2) containing 1.0% 2-mercaptoethanol and 25% glycerol at 90°C for 10 min. The treated enzyme preparations were subjected to electrophoresis in the presence of 0.1% SDS.[29] There was a single sharp band of stained protein (Fig. 1A). The molecular weight of the polypeptide in the band was calculated to be approximately 50,000±2000 from a semi-logarithmic plot of molecular weights of marker proteins against their mobilities (Fig. 1B). The results indicate that the enzyme consists of two subunits identical in molecular weight.

3. NH_2-Terminal Analysis

To obtain further informations about the molecular structure of the enzyme, NH_2-terminal amino acid analysis was carried out. Dansylation of the enzyme gave only the dansyl derivative of valine as the NH_2-terminal residue. About 1.8 moles of DNP-valine was produced per mole of enzyme with the recovery of 46% by Sanger's DNP method; the enzyme is composed of the two identical polypeptide chains.

4. Absorption Spectrum of Enzyme and Reduction with Sodium Borohydride

The holenzyme exhibits absorption maxima at 280 and 430 nm at pH 7.4 with molecular absorption coefficients of 72,000 and 10,500, respectively (*Curve A*, Fig. 2). No appreciable spectral shifts occurred by varying the pH (5.5–9.0). Reduction with sodium borohydride affects both the absorption spectrum and the enzyme activity. The enzyme was treated with 5 mM sodium borohydride at about 5°C for 10 min by the dialysis method of Matsuo and Greenberg,[30] and then dialyzed against 10 mM potassium phosphate buffer (pH 7.4). The reduction caused an irreversible loss of

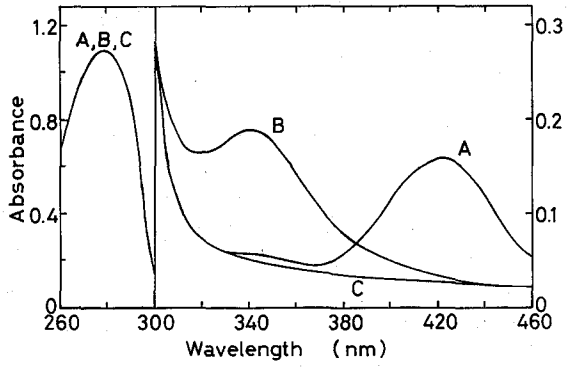

Fig. 2. Absorption spectra of fungal kynureninase. *Curve A*, holoenzyme in 10 mM potassium phosphate buffer (pH 7.4); *Curve B*, holoenzyme reduced with $NaBH_4$, in 10 mM potassium phosphate buffer (pH 7.4); *Curve C*, apoenzyme prepared as described in the text in 50 mM potassium phosphate buffer (pH 7.4).

the activity: the addition of pyridoxal-P to the reduced enzyme did not reverse the inactivation. Spectral measurements showed that the 430-nm peak diasappeared with appearance of a new peak at about 340 nm (*Curve B*, Fig. 2). This peak did not change after further dialysis against the above buffer at 4°C for about 15 h. These results suggest that the borohydride reduces an aldimine linkage (λ max, 430 nm) formed between the 4-aldehyde group of pyridoxal-P and an amino group of the protein to yield an aldamine bond (λ max, 340 nm). The amino group to which pyridoxal-P binds in the enzyme is probably an ε-amino group of lysine residue by analogy with kynureninase of *Ps. fluorescens*[16] and other pyridoxal-P enzymes thus far studied.[31]

5. Fluorescence Spectra

The fluorescence spectra of the enzyme were measured at pH 7.2 (Fig. 3). The enzyme emitted fluorescent light upon excitation at 280 and 430 nm, and the emission maxima were observed at 338 and 495 nm, respectively. When the enzyme was analyzed at 338 and 495 nm, excitation spectra exhibited the maxima at 283 and 422 nm, respectively, corresponding to the absorption maxima of the enzyme.

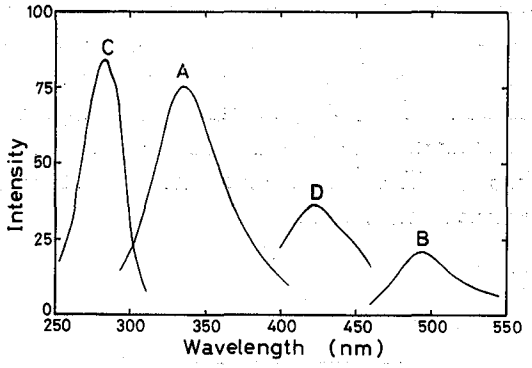

Fig. 3. Fluorescence spectra of fungal kynureninase. The enzyme was dissolved in 10 mM potassium phosphate buffer (pH 7.2). Emission spectra A and B were measured by exciting at 280 and 430 nm, respectively. Excitation spectra C and D were taken by analyzing at 338 and 495 nm, respectively.

6. Pyridoxal-P Content

Since the bound pyridoxal-P was gradually released from the holoenzyme by dialysis against deionized water, the enzyme was dialyzed thoroughly against 10 mM potassium phosphate buffer (pH 7.2) containing 5 μM pyridoxal-P. The cofactor content of the enzyme was determined in triplicate experiments with the three different enzyme samples according to the following three independent methods by deducting the contents in the dialysate from those in the dialyzed enzyme.

6.1. Penylhydrazine Method

After the enzyme was treated with 0.1 N HCl at 37°C for 30 min to release the

bound pyridoxal-P, the amount of free pyridoxal-P was determined with phenylhydrazine reagent.[32] An average value of 1.08 mole of pyridoxal-P/mole of enzyme was obtained.

6.2. KCN Method

The enzyme samples were hydrolyzed with 0.055 N HCl at 110°C for 6 h[33] and analyzed fluorometrically with KCN by the method of Bonavita.[34] The average cofactor content of 1.19 mole/mole of enzyme was obtained.

6.3. MBTH Method

The enzyme solution was treated with 0.1% MBTH in 0.3 M potassium phosphate buffer (pH 8.0) at 50°C for 30 min.[35] An average value of 0.96 mole of pyridoxal-P/mole of enzyme was given. The results obtained by these procedures show that one mole of pyridoxal-P is bound per mole of enzyme.

7. Resolution and Reconstitution of Kynureninase

Resolution of the enzyme was performed by treatment with hydroxylamine as follows. The enzyme was incubated with 10 mM hydroxylamine (pH 7.2) at 25°C for 3 h, and then applied to a Sephadex G-25 column (1 × 30 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) followed by elution with the same buffer. The enzyme had no detectable activity in the absence of added pyridoxal-P, and no longer exhibited an absorption maximum at 430 nm (*Curve C*, Fig. 2). Activity was fully restored by addition of 50 μM pyridoxal-P. The apparent Michaelis constant for pyridoxal-P was estimated to be 0.14 μM in the presence of 50 mM potassium phosphate buffer (pH 8.0). The enzyme reconstituted with pyridoxal-P showed the same spectrum as the native holoenzyme.

8. Effect of pH Activity

The pH dependence of kynureninase was determined in various buffers. The

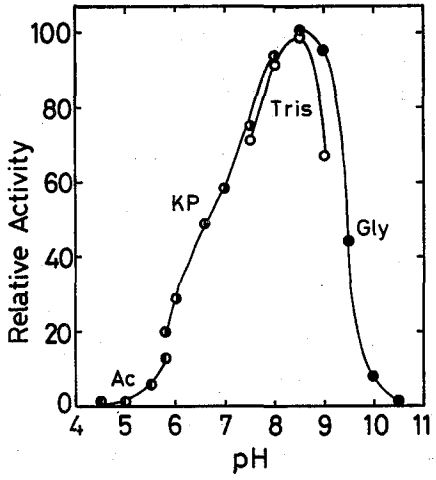

Fig. 4. Effect of pH on kynureninase activity. The enzyme activity was measured with the following buffers. Ac (◐), acetate-sodium acetate; KP (◑), potassium phosphate; Tris (○), Tris-HCl; and Gly (●), glycine-KOH-KCl.

enzyme is active in the alkaline pH region with a maximum at about pH 8.5 (Fig. 4). The concentrations of Tris-HCl buffer and glycine-KOH-KCl buffer showed no effect on the enzyme activity under the experimental conditions. The optimum pH value for the fungal inducible kynureninase is higher by 0.5 than that for the bacterial enzyme.[16]

9. Substrate Specificity and Kinetics

Although the bacterial kynureninase catalyzes almost exclusively the hydrolysis of L-kynurenine,[16] the fungal enzyme acts on both L-kynurenine and L-3-hydroxykynurenine.[20] L-3-Hydroxykynurenine is the best substrate for the fungal enzyme with a Vmax value of 5.6 μmole/min/mg of protein, while the value for L-kynurenine is 2.7 μmole/min/mg of protein. The Km values for L-kynurenine and L-3-hydroxykynurenine were determined to be 35 and 18 μM, respectively, from double reciprocal plots for the relationship between reaction velocity and substrate concentration (Fig. 5). *N'*-Formyl-L-kynurenine also can be hydrolyzed, though slowly: the rate is less than one tenth of that for L-kynurenine, and the Km value is approximately 37 μM. D-Kynurenine, L-tryptophan, L-aspartate, L-asparagine and L-glutamine were not substrates when the enzyme (180 μg) was incubated at 25°C for 60 min and assayed by measuring alanine formed with a Yanagimoto LC–5S amino acid analyzer.

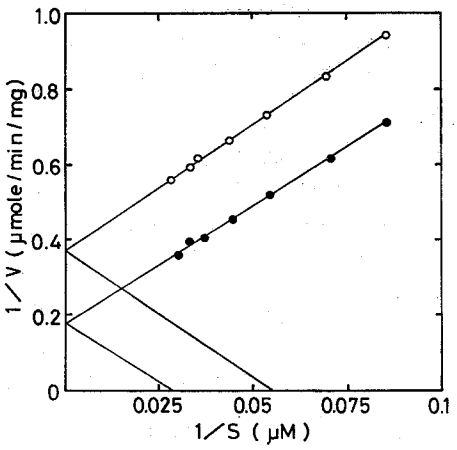

Fig. 5. Effect of substrate concentration on kynureninase activity. The activity was determined under the standard assay conditions except the indicated concentration of L-kynurenine or L-3-hydroxykynurenine. The reciprocal velocity was plotted against the reciprocal concentration of L-kynurenine (○) or L-3-hydroxykynurenine (●).

10. Inhibitors

The various compounds were investigated for their inhibitory effects on enzyme activity (Table I). The enzyme is inhibited most strongly by hydroxylamine and phenylhydrazine, typical inhibitors for pyridoxal-P enzymes. D-Cycloserine, L- and D-penicillamine, potassium cyanide, and semicarbazide also are inhibitory. Thiol

Table I. *Effect of Inhibitors on the Fungal Kynureninase Activity*

Compounds	Relative activity[a]
None	100
Hydroxylamine	0
Semicarbazide	87
D-Cycloserine	87
L-Penicillamine	45
D-Penicillamine	58
Phenylhydrazine	0
Potassium cyanide	52
$HgCl_2$	21
Iodoacetate	97
p-Chloromercuribenzoate	30
N-Ethylmaleimide	67

a) The enzyme was incubated with the compounds listed (1 mM except *p*-chloromercuribenzoate (0.5 mM)) at 25°C for 10 min. The reaction was started by addition of L-kynurenine.

reagents, *e.g.* $HgCl_2$ and *p*-chloromercuribenzoate, significantly inhibited the enzyme. These inhibitory effects of carbonyl and thiol reagents on the enzyme are similar to those for the bacterial kynureninase.[16] Ethylenediaminetetraacetic acid and divalent cations such as Mg^{2+}, Mn^{2+} and Ca^{2+} had no effect on the activity.

11. Stability of Enzyme

The crystalline enzyme loses no appreciable activity for periods of over a month when stored at 4°C as a suspension in 10 mM potassium phosphate buffer (pH 7.2) containing 50 μM pyridoxal-P and 60% saturated ammonium sulfate. When the enzyme was heated at 50°C for 10 min, it was found stable in the pH range of 6.5–8.5, while the apoenzyme was inactivated even in the neutral pH range.

DISCUSSION

Recent studies showed that *N. crassa* possesses two types of kynureninase.[19,36] The one is inducibly formed by L-tryptophan and functions biodegradatively by convering L-kynurenine to anthranilate, and the other which is termed also as hydroxykynureninase is constitutive and predominantly catalyzes the hydrolysis of L-3-hydroxykynurenine to L-alanine and 3-hydroxyanthranilate leading to the biosynthesis of NAD. Both the enzymes were partially purified by DEAE-cellulose column chromatography to show difference in their kinetic properties and in their affinities to pyridoxal-P.[19]

The studies described here deal with the characterization of the inducible-type kynureninase purified to homogeneity and crystallized from *N. crassa* grown in a tryptophan medium. The physicochemical and enzymological properties of *Neurospora* kynureninase in general are closely similar to those of the *Pseudomonas*

enzyme.[16] The fungal enzyme (Mr 105,000), composed ot two identical subnits (Mr 50,000), contains one mole of pyridoxal-P as previously demonstrated for *Pseudomonas* kynureninase.[16] This suggests that both enzymes have "half of the sites reactivity", in which only one of two apprarently identical subunits participates, at least directly, in the reaction. Half of the sites reactivity has been observed with various enzymes,[37,38] but only with L-threonine dehydrase of *Salmonella typhimurium*[39] and D-amino acid aminotransferase of *Bacillus sphaericus*[40] among pyridoxal-P enzymes. D-Amino acid aminotransferase consists of two identical subnits, one of which binds one mole of pyridoxal-P in a different way and is not directly concerned with the enzyme activity. In most of pyridoxal-P enzymes one mole of pyridoxal-P is bound per mole of subunit.[31] Although several explanations have been advanced in an effort to account for the findings,[37] further investigation is needed to elucidate significance of half of the sites reactivity in kynureninase.

Pseudomonas kynureninase shows an absorption maximum at 337 nm in addition to maxima at 280 and 430 nm, but the *Neurospora* enzyme has no appreciable peak or shoulder in the 330–340 nm region. In view of the characteristic absorption maximum at 430 nm, and also borohydride reduction of the enzyme, pyridoxal-P is bound to an amino group (probably an ε-amino group of lysine residue by analogy with other pyridoxal-P enzymes) of the protein through an aldimine linkage.

The enzyme has higher affinity to L-kynurenine than to L-3-hydroxykynurenine as reported by Gaertner *et al.*,[19] but difference between Km values for both substrates is small. In addition, the Vmax value for L-3-hydroxykynurenine is about twice that for L-kynurenine in contrast to the data previously reported.[19] The enzyme of *Neurospora* is formed inducibly several hundreds-fold by addition of L-tryptophan to the medium.[5,41] Thus, it is plausible that the inducible kynureninase functions catabolically, and does not play a role in biosynthesis of NAD and NADP.

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